Genetic Dissection of Neural Properties using Somatic Cell Hybrids

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A set of neuronal genes can be expressed in neuroblastoma × L cell hybrids and hybrid cell lines with specific defects in neural function can be generated in high yield.

THE nervous system can be genetically dissected using mutations in whole animals[1-3]. Clonal lines of cells derived from the nervous system and maintained *in vitro* also allow genetic manipulation, and provide large quantities of material for elucidating the biochemical mechanisms underlying neural function. Clones can be used to assign functions to specific cell types and to study the inheritance and stability of the differentiated phenotypes while varying the environment (refs. 4–8 and S. Brenner, lecture delivered to the Royal Society, London, on April 22, 1971). By fusing cells which differ in the expression of specific genes, it is possible to explore the mechanisms controlling gene expression[9,10] while the chromosomal alterations which occur in hybrid cells[11-13] generate genetically different cell lines.

Although most differentiated functions are extinguished in hybrid cells[14-18], neuroblastoma × L cell hybrids proved to be an exception because these cells retain the ability to synthesize electrically active membranes[19]. In this report, we extend our initial observations and show that many neuroblastoma × L cell hybrids have, as well as electrically excitable membranes, high levels of acetylcholinesterase (AChE, EC 3.1.1.7) and neurites—in some cases, more than the parent neuroblastoma cells. Moreover, hybrid clones defective in each neuronal property were found.

Neuroblastoma cells resistant to 6-thioguanine (N4TG1, N18TG2, N18TG2rc) and expressing AChE, neurites that impregnate with silver, electrically excitable membranes and regulation of these properties, were fused in the presence of inactivated Sendai virus to BrdU resistant L cells (B82), not expressing these functions (see legend for Fig. 2). Hybrid colonies were isolated from separate plates 2–3 weeks later as independent mating events in selective HAT medium[20].

The neuroblastoma × L cell (N × L) hybrid clones grew rapidly with population doubling times of <20 h in HAT medium. The hybrid nature of the N × L clones was verified in three further ways. Neuroblastoma cells derived from strain A mice and L cells derived from C_3H mice express different glucose phosphate isomerase (GPI) and phosphoglucomutase (PGM) phenotypes at the autosomal *Gpi-1* and *Pgm-1* loci when analysed by starch gel electrophoresis[19-24]. The N × L hybrid cell lines expressed both neuroblastoma (a band) and L cell (b band) GPI-1 phenotypes and a third nonparental isozyme (h band) as described for F1 animals heterozygous at this locus[22]. Most of the hybrids expressed both PGM-1 phenotypes.

Neuroblastoma and L cell lines were derived from the mouse, and therefore do not differ markedly in chromosome morphology. But neuroblastoma parent N4TG1 has an acrocentric marker chromosome, and N18TG2 and N18TG2rc have an additional minute metacentric marker chromosome not seen with the L cell parent (B82); whereas B82 has seven submetacentric marker chromosomes not found with the neuroblastoma cell lines. Submetacentric L cell marker chromosomes were found in all N × L hybrid lines and either the minute metacentric or acrocentric neuroblastoma marker chromosome were found with most of the N × L hybrid cell lines.

AChE Activity in Hybrid Cells

The maximal specific activity of AChE found with neuroblastoma cells was forty (N4TG1) and 330 times (N18TG2) greater than that found with L cells. The specific activity of neuroblastoma AChE is relatively low in the logarithmic phase of growth, but rises as the cells enter the stationary phase[7]. Because AChE is a regulated enzyme, the parent and hybrid cells were tested for AChE specific activity at various times after inoculating microwells. Neuroblastoma parent lines exhibited four (N4TG1) and twenty-two-fold (N18TG2) increases in AChE specific activity, while L cell AChE specific activity fell during the growth curve (Fig. 1).

The specific activity of AChE found with N × L hybrid clones is shown in Fig. 2*A* and *B*. Three classes of AChE expression were found: (*a*) values equal to or higher than the parental neuroblastoma clones; (*b*) values intermediate between the neuroblastoma and L cell values; and (*c*) clones with low AChE activity.

The two neuroblastoma parents yielded different types of hybrid clones. Hybrid clones with greater than parental levels of AChE were only found in the N4TG1 × B82 cross. In the second cross (N18TG2 × B82) the differences in AChE levels between hybrid clones were more pronounced. Hybrid clones were found with maximal specific activity similar to the neuroblastoma parent, while others were fifty to 100 times lower than the parent. These latter clones, however, had values five to twenty-five times greater than that expressed by the L cell parent.

The AChE specific activity in most hybrid clones was low during the logarithmic phase of growth, and increased as the cells became confluent. Clones with low levels of AChE also exhibited regulation of the enzymatic activity. For example, hybrid clones NL145, 302, 304 showed seven to nine-fold rises in specific activity of AChE (0.5→3.4, 4.6 AChE units) during the growth curve experiments.

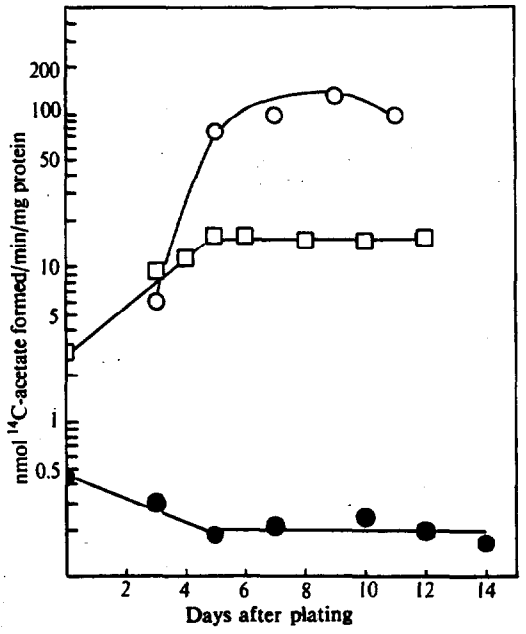

Fig. 1 Acetylcholinesterase specific activity in parental clones as a function of time after plating. Logarithmic phase cells were trypsinized and plated on day 0 at 10^4 cells per microwell (0.5 cm diameter) in 0.2 ml. of medium, and fed on days 4, 6, 8, 10, 12. Confluent monolayers developed by days 5–6. Homogenates from cells grown in microwells were prepared by removing the medium, washing the monolayer once with Puck's saline D_2[25], twice with saline D_1 (both supplemented with 59 mM sucrose and 5.6 mM glucose), then 0.075 ml. of solution A containing 0.05 M potassium phosphate (*p*H 6.8) and 0.001 M EDTA at 4° C was added and the cells were lysed by sonication in the well using a Bronwill sonicator (number 10 setting, 4 s, at 4° C). Wells were washed once with 0.025 ml. of solution A and homogenates from two wells were pooled, frozen, and stored at −190° C. In some experiments, the homogenates were prepared by collecting cells from large culture vessels as previously described[7]. AChE activity was determined by a radiometric assay[7]. Each reaction contained the following components, in a final volume of 0.05 ml.: 0.05 M potassium phosphate buffer (*p*H 6.8); 0.2 M NaCl; 1 mM EDTA; 0.5% 'Triton X-100' (Packard); 3.3 mM 1-^{14}C-acetylcholine iodide (0.39 μCi μmol^{-1}, New England Nuclear); and 0–0.4 mg homogenate protein. Each value represents the average of two to four homogenates assayed for AChE at four different homogenate concentrations, and represents AChE activity inhibited by more than 95% by 2×10^{-6} M 1,5-*bis*(4-allyldimethylammoniumphenyl)pentane-1,3-dibromide (BW284C51, Burroughs Wellcome), a more potent inhibitor of AChE than cholinesterase (ChE, EC 3118) (ref. 26). Reactions were incubated for 10 or 30 min at 37° C. In each case, the rate of reaction was proportional to homogenate concentration. Protein was determined by a modification of the method of Lowry *et al.*[27]. ○, N18TG2; □, N4TG1; ●, B82.

Homogenate mixing experiments between neuroblastoma and L cell parents revealed no inhibition of AChE activity. Nor were any significant differences in AChE specific activity detected between N18TG2 grown in medium D or in medium D supplemented with hypoxanthine and thymidine (HT), or between NL308 grown in medium D, medium D + HT, or HAT. Thus, no enzyme effectors were detected.

AChE in Parental and Hybrid Clones

The response of AChE activity from stationary phase cells to selective esterase inhibitors was similar in neuroblastoma parent and hybrid clones. The 50% molar inhibition values were: tetrammonoisopropyl pyrophosphortetramide (Iso-Ompa (Pierce Chemicals), 3×10^{-4} M; 1,5-*bis*(4-allyldimethylammoniumphenyl)pentane-1,3-dibromide (BW284C51, Burroughs Wellcome), 3×10^{-8} M; neostigmine, 1×10^{-8} M; di-isopropylfluorophosphate (DFP), 2×10^{-8} M. L cell (B82) values were different for Iso-Ompa (8×10^{-4} M); BW284C51 (2×10^{-7} M); and neostigmine (6×10^{-8} M), but this can probably be accounted for by the 10 to 50-fold increase in homogenate protein required for significant hydrolysis of the labelled acetylcholine.

The rate of hydrolysis of acetylcholine as a function of substrate concentration was also determined. Mouse neuroblastoma AChE has a peak rate of hydrolysis at $3–5 \times 10^{-3}$ M acetylcholine and inhibition at higher concentrations (S. Wilson, B. Schrier, E. Thompson, R. Rosenberg, A. Blume and M. N., manuscript in preparation). This was also true of the mutant neuroblastoma and hybrid AChE activity. The reaction velocity fell four-fold as the acetylcholine concentration was increased from 10^{-3} M to 10^{-1} M. Thus, parental neuroblastoma and N × L hybrid cell enzyme activity seems to be AChE.

Histochemical Staining

The frequency of hybrid clones expressing and not expressing AChE activity was determined by histochemical staining for AChE[28] about ten generations after fusion while the hybrid cells were still in their original colonies. Further, fidelity of transmission of the neuronal phenotype from one cell generation to the next was tested by staining many parental colonies for AChE. Parental cells were plated at 200–500 cells/60 mm dish and the resultant colonies were stained 14–18 days later. All 6,538 parental neuroblastoma colonies (N18TG2, N18TG2rc, N4TG1) stained positively for AChE, giving a reversion frequency for this neuroblastoma marker of $< 1.5 \times 10^{-4}$ per colony forming unit. None of the 8,053 L cell (B82) colonies tested stained positively. Of the 1,911 hybrid clones tested approximately 40% were (+) and 60% (−) (Table 1), and the recloned neuroblastoma parent (N18TG2rc) yielded the two classes of hybrids with respect to AChE staining. Thus, both classes of hybrid clones (AChE+ and −) appeared in the early

Table 1 Histochemical Stain for AChE in N × L Hybrid Colonies Ten Generations after Fusion

N × L cross	Parental growth conditions before fusion	% AChE (+)	P*	Total No. of colonies tested	Hybrid colonies per input neuroblastoma cell × 10^4
N18TG2rc × B82	Log × S	39	<0.05	223	6.2
N18TG2rc × B82	S × S	47	<0.001	702	9.4
N18TG2rc × B82	S × M	66		232	11.0
N4TG1 × B82	S × S	51	<0.001	168	2.3
N4TG1 × B82	S × S (1 : 200)	21		86	19.1
N18TG2 × B82	S × S	26	<0.001	497	6.5

* The P values indicate statistical comparison by chi square test between the bracketed values. The value for N18TG2 results from comparison with N18TG2rc, and N4TG1 in the S × S crosses.

AChE histochemical staining of colonies was performed using the method of Karnovsky and Roots[28], modified by adding 10^{-4} M Iso-Ompa, an inhibitor of ChE. Colonies were washed with phosphate buffered saline (PBS (*p*H 7.4) 340 mosm l.$^{-1}$), fixed for 10 min in PBS with 10% (v/v) formalin at 4° C, washed with PBS, and then with the staining solution minus substrate. The colonies were incubated with the staining solution for 3–5 h at 37° C, washed with distilled water, counted and scored. AChE(+) colonies had a red-brown precipitate in their cells while AChE(−) colonies did not. Precipitate formation was dependent on acetylthiocholine iodide or acetyl-β-methylthiocholine iodide (Sigma)[29], and inhibited by 10^{-4} M BW284C51. Crosses were performed at neuroblastoma : L cell ratio of 1 : 20, except the cross indicated 1 : 200. Parental cells were in the following phases of growth prior to the crosses: S, stationary phase; Log, logarithmic phase; M, L cell population enriched for mitotic cells by harvesting, without trypsin, round, loosely attached L cells from a rapidly dividing population. One day after fusion the reaction mixtures were trypsinized, diluted and replated in HAT medium to give less than twenty hybrid colonies per 60 mm plate. The hybrid colonies were stained 18–25 days after fusion (10^3 cells per colony). The probability that any one colony represented a revertant neuroblastoma cell ranged from 2.2×10^{-3} to $< 0.5 \times 10^{-4}$ (calculated by multiplying the reversion frequencies of the neuroblastoma parents in HAT medium by the number of input neuroblastoma cells divided by the number of hybrid colonies formed).

generations, and arose from neuroblastoma cells unequivocally derived from a single progenitor cell.

The frequency of (+) colonies was related to the parental neuroblastoma clone used and to conditions imposed at the time of fusion. Significant differences in the fraction of (+) colonies appeared when : (1) logarithmic rather than stationary phase neuroblastoma cells were fused to stationary phase L cells; (2) when stationary phase neuroblastoma cells were fused with an L cell population enriched for mitotic rather than stationary phase cells; (3) when the ratio of L to neuroblastoma cells was increased; and (4) when N18TG2 was compared with either N4TG1 or N18TG2rc (Table 1).

Neurite Formation in Hybrid Cells

Neuroblastoma cells extend branched processes that impregnate with silver stains (neurites) whereas L cells do not[6]. The available evidence suggests that silver impregnation is correlated with the development of neurofibrils in the processes[30]. Most N×L hybrid clones had extensive process formation in the early generations after fusion as well as in later generations. Cell lines scored as positive for process formation had more than 50% of the cells in one of the test conditions extend processes of >100 μm, while process negative cell lines had less than 0.1% of the cells develop long processes under any condition (Figs. 3 and 4). Three classes of neurite formation by hybrid clones were seen: (*a*) long (>100 μm), branched processes that formed networks and impregnated with silver using the method of Bodian[31] (Figs. 3*C*, 4*B*, 5); (*b*) long, thin processes with rare branching but no silver impregnation (Fig. 4*D*); and (*c*) no process formation (Fig. 3*D*). Of interest, most hybrid clones exhibited more extensive process formation than the neuroblastoma parental cell lines. In most hybrid clones the fraction of cells with processes increased after incubation in medium minus serum (a result similar to that previously described for neuroblastoma cells[6]). Several of the hybrid clones, however, showed extensive process formation in the presence of serum (Fig. 4).

Electrical Properties of Hybrid Cells

Neuroblastoma cells exhibit electrically excitable membranes following depolarizing stimuli while L cells do not[19,32]. Using intracellular microelectrode recordings the membrane voltage curves following depolarization of the hybrid cells were scored for the presence or absence of inflexions on the rising phase

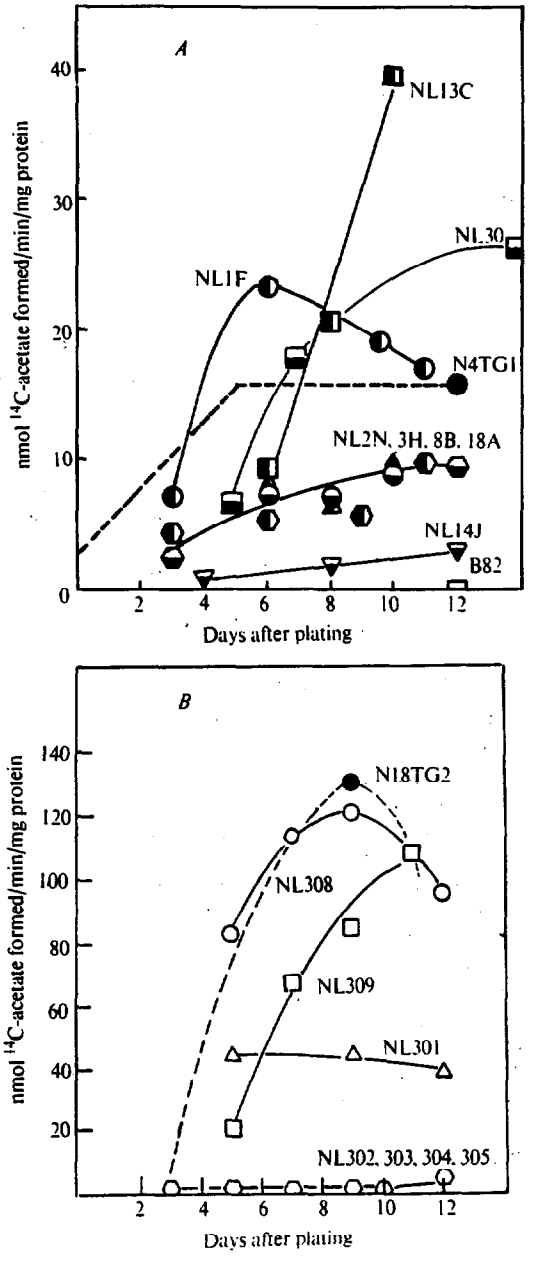

Fig. 2 Acetylcholinesterase specific activity in N×L hybrid clones as a function of time after plating. Logarithmic phase cells were plated (10^4 cells/microwell) in HAT medium on day 0 and confluent monolayers developed by days 5–6. Homogenates were prepared and AChE specific activity determined as in Fig. 1. *A*, N×L hybrids from the cross N4TG1×B82. *B*, N×L hybrids from the cross N18TG2×B82. The dotted lines indicate neuroblastoma parental values during the growth curve. Clones N4TG1 and N18TG2 unable to utilize exogeneous hypoxanthine were derived from mouse neuroblastoma C1300 clones N4 (ref. 19) and N18 (ref. 6) by ethyl methane sulphonate mutagenesis followed by sequential selection in 10^{-6} M and 10^{-4} M 6-thioguanine (Sigma) and subsequent recloning in 6-thioguanine as previously described[19]. Subclone N18TG2rc was derived from N18TG2 by visually identifying single cells in microwells (Falcon). The neuroblastoma mutants grow in 10^{-4} M 6-thioguanine but do not survive in growth medium supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin (a gift of Lederle Pharmaceutical Co.), and 1.6×10^{-5} M thymidine (HAT medium)[20]. Colony forming efficiencies in HAT medium after growth in 6-thioguanine were: N4TG1 ~1–5×10^{-7}, N18TG2 and N18TG2rc $<1\times10^{-7}$. However, cells in "revertant" colonies grew slowly and with low plating efficiency. Mutant L cell clones B82, deficient in thymidine kinase and A9, deficient in hypoxanthine phosphoribosyl transferase were the gift of Dr J. Littlefield[21]. Dulbecco's modification of Eagle's medium (GIBCO) and 5% foetal calf serum (Colorado Serum Co.) without antibiotics (medium D), in an atmosphere of 90% air–10% CO_2 in Falcon flasks, dishes, or microwells was used. Parental cell lines were grown in medium D containing the compounds used to select for the enzyme defects for 7 days before fusion (10^{-4} M BrdU (Sigma) for B82, and 10^{-4} M 6-thioguanine for N4TG1, N18TG2, N18TG2rc, and A9). Parental cells from confluent cultures were mixed in varying ratios (1 : 20 to 1 : 200, neuroblastoma : L cell) in 60 mm plates (2×10^6 cells/plate) and fused with β-propiolactone inactivated Sendai virus (500–1,000 haemagglutinating units) as previously described[19]. One day after fusion, cells were dissociated with 0.05% trypsin (Difco 1 : 250), diluted and plated into 60 mm Petri dishes or directly into microwells in HAT medium. Clones were isolated in three ways: (1) well isolated colonies were picked with Pasteur pipettes from plates, grown for more than twenty generations, recloned by plating less than 0.8 cells/microwell, allowing the cells to attach, and then microscopically selecting those wells containing only one cell; (2) fusion mixture were diluted and plated directly into microwells to give less than 0.5 hybrid colonies/well, and wells developing only one colony were identified; (3) well isolated colonies arising in separate Petri dishes were picked with Pasteur pipettes. All the clones arising from the crosses N4TG1×B82 and N18TG2×B82 were isolated by methods 1 or 2. Clones from the cross N18TG2rc×B82 were isolated by method 3. For all studies parental cells were grown in medium D while the hybrid lines were grown in medium D supplemented with HAT. Cells were frozen in medium D supplemented with 7% dimethylsulphoxide (Sigma) and stored in the vapour phase of a N2 freezer. Cell lines were assayed for PPLO by culture methods and by autoradiography after incubation with ^{3}H-thymidine. Experiments were performed only with cells judged to be free of PPLO.

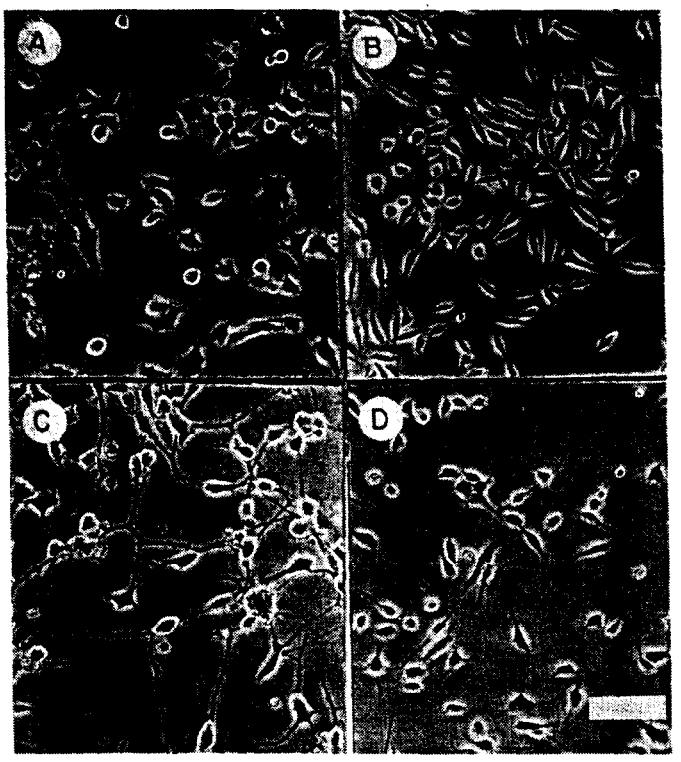

Fig. 3 Process formation in parental and hybrid cells. Cells were incubated in medium minus serum for 5 days. *A*, Parent neuroblastoma, N4TG1 (process +); *B*, parent L cell, B82 (process −); *C*, hybrid NL1F (process + +); *D*, hybrid NL13C (process −). Compare the short, spiny processes of the neuroblastoma parent N4TG1 with the long processes of NL1F. The white bar in frame D represents 100 μm.

Table 2 Responses of Cells to Electrical Stimulation

Hybrid cell line	No. of cells in response class: Passive A^-B^-	Electrically active A^-B^+	Electrically active A^+B^+ A^+B^-	Total cells studied	% Electrically active	Average resting membrane potential (mV)
N4TG1 × B82						
NL1F	1	3	14	18	94	25
NL8B	13	2	7	22	41	30
NL13C	1	13	0	14	93	19
NL14J	9	7	0	16	44	21
NL7AC	16	3	0	19	16	26
N18TG2 × B82						
NL308	3	3	6	12	75	25
NL309	14	4	4	22	36	21
NL304	4	17	0	21	81	25
NL305	13	0	0	13	0	26

Hybrid cells from confluent cultures were plated (2×10^6 cells/60 mm dish) into medium D containing 4×10^{-7} M aminopterin without hypoxanthine or thymidine, and studied 5–7 days later. This procedure selects for nondividing cells (~10% of the starting population) which retain the ability to divide if hypoxanthine and thymidine are added to the medium. Wild type neuroblastoma exhibits highly excitable membranes under these conditions[34]. Some clones in HAT medium were studied 5 days after reaching confluency in 60 mm dishes. Membrane voltage curves as a function of time after depolarizing stimulation were photographed from the oscilloscope trace and scored for the presence of an inflexion on the rising portion of the curve (A response) and an inflexion later in the trace (B response) as previously described[19]. The B^+ response was only scored in the absence of an A^+ response. No attempt was made to distinguish between A^+B^+ and A^+B^- cells. All cells were tested for depolarizing responses over a range of membrane potentials (resting potential to −80 mV), and amount of stimulating current (0–5 nA). Only cells with a stable membrane potential (>75%) were studied.

(A response) and the falling phase (B response). The available evidence indicates that the A response results, at least in part, from Na^+ entry into the cell and the B response from the exit of K^+ from the cells[33]. Clonal differences in membrane phenotypes were found (Table 2). Some hybrid clones had cells exhibiting both A and B responses ($A^+B^\pm$ and A^-B^+), some had cells with only B responses (A^-B^+), while other clones showed neither response (A^-B^-). Within a hybrid clone demonstrating electrically excitable membranes ($A^+B^\pm$ or A^-B^+), passive cells (A^-B^-) were also found indicating regulation of action potential components. Hybrid cells studied for about ten generations after fusion from the cross N18TG2rc × B82 were also electrically active ($A^+B^\pm$, A^-B^+).

Karyological Analysis

Reduction in chromosome number was observed in N × L hybrid cells. Hybrid clones with fewer total, metacentric and submetacentric chromosomes were found (Table 3). At this point, no correlation of phenotype with specific chromosomes can be made. Some clones had more than the expected number of chromosomes from fusing two average parental cells. Of these, only clone NL302 had more than the expected number of parental marker chromosomes (ten rather than seven L cell marker chromosomes).

Hybrid clones arising from the crosses N18TG2 × B82 and N18TG2rc × B82 were divided into two groups with respect to AChE: those expressing high levels of AChE (+ clones >40 AChE units) and those expressing low levels of AChE (− clones, ≤5 AChE units). Both the radiometric assay and histochemical stain were used to detect AChE and clones were then analysed karyologically at approximately the same number of generations after fusion (twenty to forty; Tables 3 and 4). AChE(+) clones had in general more chromosomes than AChE(−) clones. The number of metacentric and submetacentric chromosomes was similar in the two groups. In both

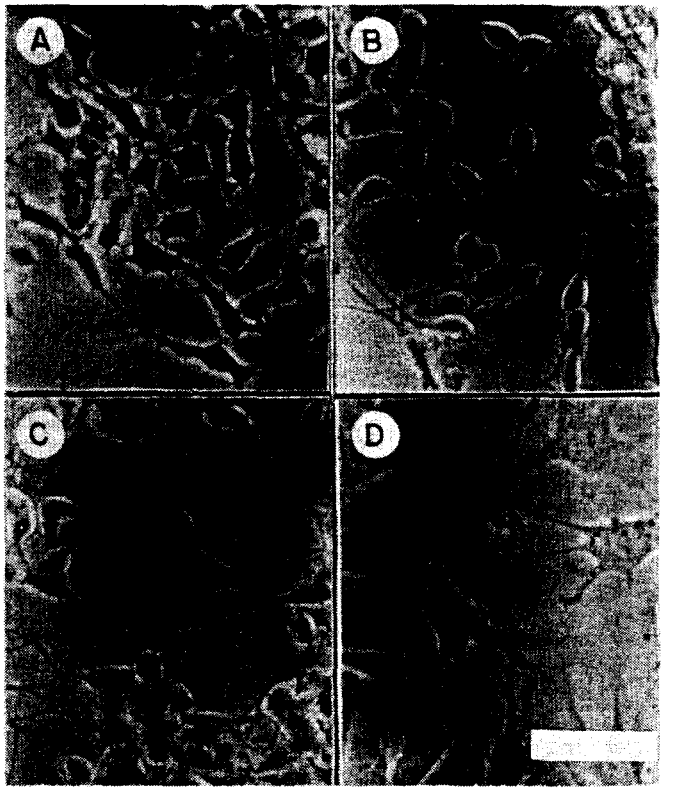

Fig. 4 Process formation in parental and hybrid cells. Cells were incubated in complete medium. *A*, Parent neuroblastoma, N18TG2 (process +); *B*, hybrid NL309 (process + +); *C*, edge of hybrid colony ~10 generations after fusion from the cross N18TG2rc × B82 (process +). White bar in Frame D represents 100 μm.

groups, hybrid clones with fewer than the expected number of chromosomes were found.

Phenotypic Classes of N × L Hybrid Cells

Scoring hybrid clones for expression of AChE, neurite formation, and electrical activity revealed seven distinct phenotypic classes (Table 5). N × L hybrid cell lines defective in one or more of the properties studied, generally had a lower total number of chromosomes than lines expressing all of the properties, suggesting that the variant lines arose by chromosomal segregation.

These results show that neural functions can be dissected with N × L hybrids. Each phenotype is clonally inherited and therefore probably is a consequence of differences in cell genome. The frequency of variant phenotypes found (ten out of sixteen clones) should be compared with the relatively stable phenotype of the parent neuroblastoma cells. While N × L crosses are intraspecific, hybridization between species may increase the generation of variants through an enhanced rate of chromosomal segregation[11].

Neuronal Maturation

The dissection of neuronal properties provides information on the number and sequence of steps occurring in neurone maturation. For example, a cross correlation of the properties (Table 6) suggests that some but not all of the neuronal properties studied can be expressed independently. One point of interest derived from such a correlation is that action potential step B can be expressed independently of step A, neurite formation, and high AChE levels, while action potential step A was not expressed independently of these properties. Similarly, high levels of AChE can be expressed independently of neurite formation and action potential step A. Such data also suggest a possible sequence of steps in neurone maturation: Neuroblast→step B, process formation→AChE→Bodian stain (neurofibrils)→step A.

Table 3 Cell Phenotype*

Cell line	AChE† units	AChE‡ regulation	Process formation§	Bodian stain	Membrane response to electrical stimulation A+	Membrane response to electrical stimulation B+	Total	Mean chromosome No. Metacentric	Mean chromosome No. Submetacentric**	No. metaphases scored
Parental										
N4TG1	16	+	+	+	+	+	142	7	0.4	21
N18TG2	131	+	+	+	+	+	89	8	<0.05	20
B82	0.2	−	−	−	−	−	52	20	7.2	29
A9	0.1		−	−	−	−				
Hybrid										
N4TG1 × B82							194	27	7–8 (Expected‖)	
NL13C	40	+	−	−	−	−	111	14	5	21
NL30	27	+	+	−			135	21	7	17
NL1F	26	+	++	+	+	+	151	21	9	14
NL7AC	12	+	++	+	−	+	120	15	5	22
NL2N	10	+	+	−			85	18	6	14
NL3H	10	+	+	−			138	14	4	12
NL18A	10	+	+	+			183	22	7	16
NL8B	9	+	++	+	+	+	158	24	8	18
NL14J	3	+	++	−	−	+	124	12	4	15
N18TG2 × B82							141	28	7 (Expected‖)	
NL308	122	+	++	+	+	+	115	24	7	26¶
NL309	108	+	++	+	+	+	129	25	7	15
NL301	47		++	+			155	27	7	16
NL302	5	+	+	−			139	41	10	14
NL304	5	+	−	−	−	+	97	25	7	17
NL303	2		−	−			57	20	6	20¶
NL305	1	−	+	−	−	−	102	25	6	21
B82 × A9										
LL-1	0.2		−	−	−	−				

All hybrid cell lines were generated from an N : L fusion ratio of 1 : 20 except : NL2N, 7AC, 8B, 18A, where the N : L ratio was 1 : 200. Hybrids from the N4TG1 × B82 cross were all tested more than 100 generations after fusion, except NL30, which was tested after forty generations. Hybrids from the N18TG2 × B82 cross were all tested after more than forty generations, except NL309, which was tested more than eighty generations after fusion. LL-1 was tested about forty generations after fusion.

* Blanks indicate not tested or uncertain.

† One unit = 1 nmol ^{14}C-acetate formed/min/mg protein; values represent maximal AChE specific activities obtained during growth curve experiments with confluent cells.

‡ Indicates a rise in AChE specific activity as the cells shift from the logarithmic to the stationary phase of growth (that is, became confluent). Both neuroblastoma and L cells are not contact inhibited and some cell division occurs in both parental cell types when the cultures became confluent.

§ Scoring symbols for process formation indicate extent of formation: +, process formation; ++, extensive, branched process formation.

‖ The expected number of chromosomes of each type if two average cells are fused.

¶ Bimodal populations. The mean chromosome numbers for the second mode are, for NL308, total, 172; metacentric, 29; submetacentric, 7; and for NL303, total, 109; metacentric, 27; submetacentric, 7.

** The submetacentric chromosomes are of L cell origin, although rare N1862 and N18TG2rc metaphases will have a single submetacentric chromosome.

Mechanisms Responsible for Phenotype

The results with N × L hybrids contrast with the frequently reported extinction of differentiated functions found with other hybrid cell types[14–18]. The existence of at least one clone (NL305) defective in AChE, Bodian stain, and action potential components, raises the possibility of pleiotropic regulation of neuronal properties. We do not know whether the expression of neural properties and the existence of different phenotypic classes in N × L hybrid cells are due to gene dosage effects, loss of genes, mutation, changes in regulatory events, or genetic variability within parental clones. Neuroblastoma cells have more chromosomes than L cells; but, the relation of chromosome number to amount of specific genetic information is unclear. Difference in amounts of parental isozymes may be one way to detect unequal parental gene dosage within the hybrid cells. For example, hybrid clones NL308 and 309 (with high levels of AChE) exhibited less intense staining of the L cell GPI isozyme (band b) than the neuroblastoma band (band a) or the intermediate (band h) isozymes. Other clones with high, intermediate, or low AChE levels, however, exhibited equal staining intensity of all three bands. Chromosome loss has occurred in N × L hybrids and segregation of regulator or structural genes as well as nonchromosomal units of inheritance must be considered. Hybrid cells expressing higher levels of AChE, more electrically active membranes and more extensive process formation than the neuroblastoma parental clones were also noted. These might arise by complementation, activation of L cell genes, segregation of regulating genes of neuroblastoma origin, or represent parental heterogeneity.

Many genes may be expressed only at certain times during the cell cycle, hence clonal but unphased populations may be heterogeneous with respect to gene expression. The different phenotypes found in hybrid cells may be an expression of this heterogeneity. Fusion of neuroblastoma and L cells in different parts of the cell cycle or at different steps in the transition from neuroblast to neurone may lead to different heritable states of gene expression in the resulting hybrid clones.

The most important conclusions to be drawn from this work are that neuronal properties such as AChE, neurites, and electrically excitable membranes can be synthesized in N × L hybrid cells and that hybrid clones with specific defects in gene expression can be obtained in high yield. Such defective cell lines provide a useful system for unravelling the biochemistry of neural function. The results also suggest that normal neurones fused with established cell lines may yield clonal lines of cells expressing various neural functions. It is likely that other complex neural properties such as synapse and network formation can be genetically dissected using the somatic cell hybrid approach.

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Table 4 Correlation of Chromosome Number with AChE Phenotype in N18TG2rc × B82 Hybrid Clones

Cell line	AChE stain*	Mean chromosome No. Total	Meta-centric	Submeta-centric	No. metaphases scored
N18TG2rc	+	90	9	< 0.08	13
Expected for N18TG2rc × B82†		142	29	7.2	
NL404	+	140	27	7.2	13
NL403	+	136	30	4.9	10
NL409	+	125	25	3.4	11
NL401	+	122	24	7.2	15
NL408	+	121	23	5.3	10
NL406	−	112	21	4.9	15
NL407	−	103	30	9.1	16
NL402	−	94	21	3.1	12
NO405	−	83	22	5.3	13
Summary		Range of mean values			No clones scored‡
N18TG2 × B82 and N18TG2rc × B82 Hybrids	+	115–155	23–30	3–7	8
	−	83–112	21–30	3–9	7

* Histochemical staining of cells was carried out as described above, except acetyl-β-methylthiocholine iodide, a substrate hydrolysed more rapidly by AChE than ChE[29] was used.

† The expected number of chromosomes of each type if two average cells (N18TG2rc × B82) are fused.

‡ Hybrid NL302 representing a 2 : 1 L cell : neuroblastoma was excluded. For the two bimodal clones the lower mode was used for the AChE(+) clone (NL308), and the higher mode for the AChE(−) clone (NL303).

Table 5 Phenotypic Classes Detected in N × L Hybrid Clones*

N × L hybrid clones	AChE Level	AChE Regulation	Neurite formation Process formation	Neurite formation Silver impregnation	Electrical excitability B+	Electrical excitability A+	Electrical excitability Regulation	% of expected No. of total chromosomes†
1F, 8B, 308, 309	+	+	+	+	+	+	+	78, 81, 81 (122), 91
7AC	+	+	+	+	+	−	+	62
2N, 30, 3H	+	+	+	−				44, 70, 71
13C	+	+	−	−	+	−	+	57
14J, 304	−	+	+	−	+	−	+	64, 69
305	−	−	+	−	−	−	−	73
303	−		−	−				40 (77)

* Blanks represent not tested or uncertain.

† The figures in parentheses indicate the percentage of the total number of chromosomes of the second mode of a bimodal population.

Table 6 Requirement for Property Expression in N × L Hybrids

(X) Property expressed	Properties concomitantly expressed (y) B step	Processes	AChE	Bodian stain	A step
B step		−	−	−	−
Processes	−		−	−	−
AChE	+	−		−	−
Bodian stain	+	+	+		−
A step	+	+	+	+	

The table logic is as follows: (+), if property (x) was expressed, then property (y) was not always expressed concomitantly; (—), if property (x) was expressed, then property (y) was not always expressed concomitantly. As an example: if high levels of AChE were detected, then the B step of electrical excitability was always found, but process formation, Bodian's stain, and the A step were not necessarily found.

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